A comparative study on the effect of subtherapeutic tylosin administration on select feral or domestic porcine gut microflora grown in continuous-flow culture

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Abstract

Continuous flow cultures of feral (culture FC) and domesticated (culture RPCF) pig gut microflora were established in steady state. Cultures, in duplicate, were continuously infused subtherapeutic (25 µg/ml) levels of tylosin and sampled at intervals to assess effects on total culturable anaerobes, *Bacteroides* spp. and *Enterococcus* spp. via plating on serial 10-fold dilutions to anaerobic Brucella blood agar, Bacteroides bile esculin agar, and M Enterococcus agar supplemented without or with 100 µg tylosin/ml, the later to assess bacterial sensitivity to tylosin. Concentrations of total culturable anaerobes within culture FC decreased ($P < 0.05$), albeit slightly, following 7 days tylosin administration. Concentrations of *Bacteroides* and *Enterococcus* decreased ($P < 0.05$) to near or below detectable levels (1.0 $\log_{10}$ CFU/ml) in culture FC following 7 days tylosin administration, and tylosin-insensitive colonies were recovered at low numbers ($\leq$ 2 $\log_{10}$ CFU/ml) and did not persist. In contrast, concentrations of total culturable anaerobes, *Bacteroides* and *Enterococcus* in culture RPCF, while initially decreased upon initiation of tylosin administration, began to increase ($P < 0.05$) by as early as 4 days thereafter, with tylosin-insensitive colonies recovered as one of predominant populations. The results of this study illustrate that under the conditions of this test, subtherapeutic administration of tylosin promoted the enrichment of tylosin-insensitive bacterial populations (capable of growing on media supplemented with 100 µg tylosin/ml) within RPCF cultures (originating from a traditionally reared domesticated pig) but not from FC cultures (originating from a feral pig).

Introduction

Macrolide antibiotics are commonly used in human and veterinary medicine, primarily to treat infections caused by Gram-positive bacteria and also as a feed additive to improve production efficiency in swine (Gaynor and Mankin, 2003). Resistance can occur via acquisition of *erm* methyltransferases, which catalytically inactivate the macrolide's targeted binding site, via acquisition of multidrug efflux pumps or even, albeit infrequently, via point mutations in the microbe's genome (Chopra and Roberts, 2001; Gaynor and Mankin, 2003; Karlsson et al., 2004; Poole, 2005). Recovery of bacteria harbouring *erm* genes from domestic swine and swine production habitats is not uncommon (Chee-Sanford et al., 2001; Wang et al., 2005) but less is known regarding the quantitative acquisition and selection of resistance in bacteria, particularly within swine not reared traditionally (Stanton and Humphrey, 2004). Continuous flow culture of intestinal microorganisms has been used to study competitive interactions between commensal and pathogenic microflora (Harvey *et al.*, 2002; Hume *et al.*, 2001; Nisbet *et al.*, 2000) as well as to investigate potential factors affecting spontaneous acquisition of antibiotic resistance (Kim *et al.*, 2005). In this study, a continuous flow chemostat model established with mixed populations of porcine gut bacteria was used to assess the effects of subtherapeutic tylosin administration on select populations of resident bacteria.

Materials and Methods

Two separate mixed populations of porcine gut bacteria were established in continuous flow culture as previously described (Harvey et al., 2002; Hume et al., 2001). The RPCF culture had been previously established with cecal contents obtained from a traditionally reared pig and its initial characterization has been reported previously (Harvey et al., 2002). The other culture, defined as FC, was established under similar conditions except with cecal contents from an

adolescent feral boar killed near Caldwell, Texas, USA, approximately 2 to 4 h prior to necropsy. Both parent cultures of RPCF and FC were established and maintained in BioFlo chemostats (New Brunswick Scientific Company, Edison, NJ) with a culture volume of 550 ml. The culture medium was Viande Levure broth which was prepared and maintained anaerobically under a stream of O_2-free CO_2 and infused at 0.40 ml/min which corresponds to a 24 h vessel turnover. Cultures were incubated at 39°C and agitated at 100 rpm. Once established in steady state, initial characterization of culture FC was accomplished using traditional bacteriological culture methodologies and antibiotic susceptibility testing was performed as described in the National Committee for Clinical Laboratory Standards (now known as the Clinical and Laboratory Standards Institute [CLSI]) (NCCLS, 2004). Both parent cultures were used to provide inoculum to establish separate RPCF and FC cultures, in duplicate, which after at least 14 vessel turnovers, were continually infused with culture medium containing 25 µg tylosin/ml. Fluid samples collected immediately before and during tylosin infusion were quantitatively cultured, via plating of 10-fold serial dilutions, to the following media, each prepared with or without 100 µg tylosin/ml: anaerobic Brucella blood agar and Bacteroides bile esculin agar (Anaerobe Systems, Morgan Hill, CA), for detection of total anaerobes and *Bacteroides* spp., respectively, and M Enterococcus agar (Becton Dickinson and Company, Sparks, MD) for detection of *Enterococcus* spp. Inoculated media were incubated 48-72 h at 37 °C and colonies propagated with and without tylosin selection were enumerated. Specific identification of bacteria from select colonies was achieved using rapid ID 32 STREP, rapid 20E, 20NE, 20A, and rapid ID 32 A identification strips (bioMérieux, Hazelwood, MO). Indole spot tests (Anaerobe Systems, Morgan Hill, CA), E-test™ (AB Biodisk, Piscataway, NJ) and gas chromatography were also used in this analysis. Samples containing no detectable colonies of bacteria were given a value of 1.0 $\log_{10}$ CFU/ml. Log_{10} transformations of bacterial concentrations obtained from duplicate cultures were analyzed for main effects of day, culture type (i.e., RPCF or FC culture) and the possible interaction using a repeated measures analysis of variance (Statistix®8 Analytical Software, Tallahassee, FL, USA). Multiple comparison of means was accomplished using a Tukeys procedure.

Results and Discussion

Characterization of microflora in culture FC. Culture FC achieved steady state after 14 days continuous flow culture and the bacteriological composition was found to include *Streptococcus bovis*, *Proteus mirabilis*, *Staphylococcus epidermidis*, *Alcaligenes denitrificans*, and members of *Bacteroides*, *Lactobacillus*, *Enterococcus*, and *Clostridium*. *Campylobacter and Salmonella* were never detected in the feral culture and *E. coli* that was initially isolated from the cecal contents was never recovered once the culture had achieved steady state. *Enterococcus hirae*, *Streptococcus bovis*, *Proteus mirabilis*, *Staphylococcus epidermidis*, *Alcaligenes denitrificans* were susceptible to tylosin and erythromycin; *Bacteroides uniformis* and *Bacteroides stercoris* were resistant to gentamicin, ciprofloxacin, ceftriaxone and ampicillin. *Clostridium hathewayi* showed resistance to tylosin at MIC >512 µg/ml and was the predominant anaerobe recovered on anaerobic Brucella blood agar.

Effect of tylosin on select bacterial populations. Recovery of anaerobes from non-selective Brucella blood agar was not affected ($P > 0.05$) by culture; however, main effects of day and day by culture interaction were observed ($P < 0.01$) on recovery of anaerobes from nonselective Brucella blood agar due to a decrease in anaerobes in the FC and an increase in RPCF cultures (Figure 1A). Main effects of culture, day and a day by culture interaction were observed ($P < 0.01$) on recovery of anaerobes from tylosin-selective Brucella blood agar due to a temporary increase in tylosin-insensitive anaerobes, which were prominent even before tylosin administration, from the FC culture and a gradual enrichment of tylosin-insensitive anaerobes from the RPCF culture (Figure 1A). The prominent tylosin-insensitive anaerobe was *Clostridium hathewayi*. Main effects of culture, day and a day by culture interaction were observed ($P < 0.05$) on recovery of *Bacteroides* from Bacteroides bile esculin agar supplemented with or without tylosin (Figure 1B) due to higher *Bacteroides* concentrations in the RPCF cultures prior to administration of tylosin and to an enrichment of tylosin-insensitive *Bacteroides* spp. beginning by day 3 of tylosin administration (Figure 1B). In the case of total anaerobes and *Bacteroides*, tylosin-insensitive populations were prominent in RPCF cultures even before initiation of tylosin administration. Conversely, tylosin-insensitive *Enterococcus* spp., were not apparent prior to tylosin administration; however, upon

initiation of treatment recovery was highly variable between the two RPCF cultures regardless of culturing on M Enterococcus agar supplemented with or without tylosin. This indicates that the two RPCF cultures contained markedly different enterococcal populations. For instance, even though mean concentrations of tylosin-insensitive *Enterococcus* recovered from the RPCF cultures began to increase markedly beginning 4 days after initiation of tylosin administration (Figure 1C) this increase occurred in only one of the cultures. As a consequence, main effects of culture, day or day by culture interactions on quantitative recoveries on M Enterococcus agar supplemented with or without tylosin were not observed ($P > 0.05$) (Figure 1C).

In conclusion, results from this study revealed that subtherapeutic administration of tylosin promoted the enrichment of tylosin-insensitive bacterial populations (capable of growing on media supplemented with 100 µg tylosin/ml) within RPCF cultures (originating from a traditionally reared domesticated pig) but not from FC cultures (originating from a feral pig).

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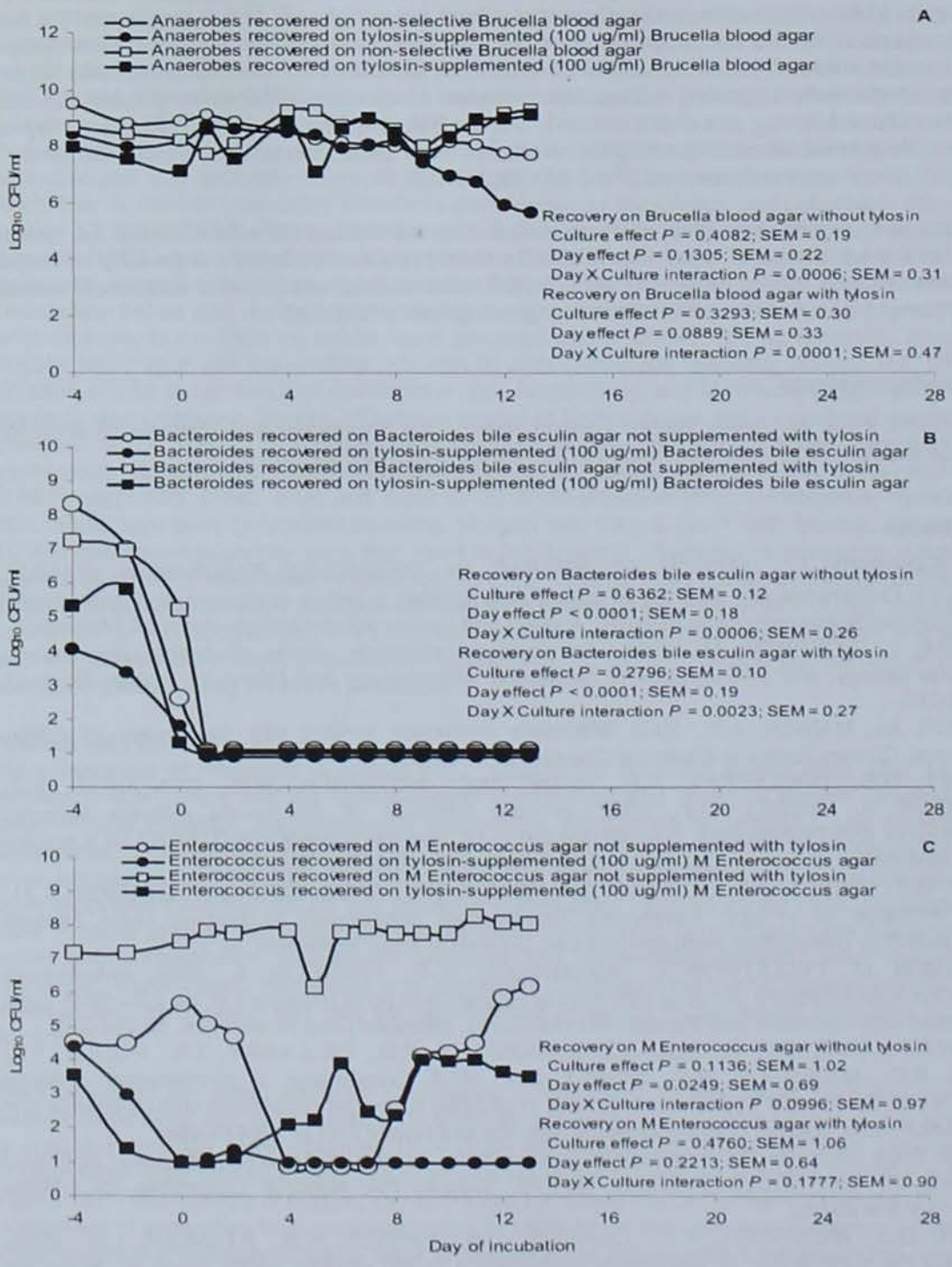

Figure 1. Effects of subtherapeutic (25 µg/ml) tylosin administration on populations of anaerobes, *Bacteroides* spp., and *Enterococcus* spp. from continuous flow cultures of mixed populations of porcine gut bacteria obtained from feral (culture FC, circles) or domestic (culture RPCF, squares) swine. Bacteria were quantitatively recovered on anaerobic Brucella blood agar, Bacteroides bile esculin agar and M Enterococcus agar each supplemented without (open symbols) or with (closed symbols) 100 µg tylosin/ml.